Purification and characterization of a Montana RMV-like isolate of barley yellow dwarf virus
by Susan Mary Geske

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
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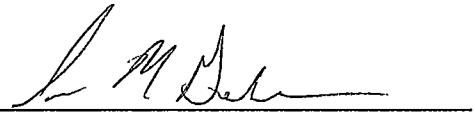
Signature 

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TABLE OF CONTENTS

	Page
LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS	x
ABSTRACT	xi
1. INTRODUCTION	1
2. BIOLOGICAL INVESTIGATION	10
Introduction	10
Materials and Methods	12
Host Plant	12
Aphids	12
Virus	13
Virus Purification and Assay	14
Estimation of Virion Chemical Components	18
Thin Section Electron Microscopy of Infected Plant Tissues	20
Polyclonal Antisera Production	21
Monoclonal Antibody Production	22
Results	24
Virus Purification and Assay	24
Estimation of Nucleic Acid Size	26
Estimation of Coat Protein Size	26
Cytopathology of Infected Tissues	26
Preliminary Evaluation of Polyclonal Antisera	35
Preliminary Evaluation of Monoclonal Antibodies	38
Discussion	40
3. MOLECULAR INVESTIGATION	48
Introduction	48
Materials and Methods	51
Nucleic Acid Isolation	51
DNA Primers	51
cDNA Production	52
PCR	54
PCR Product Analysis	54
Hybridization Analysis	55
DNA Cloning and Sequencing	56

TABLE OF CONTENTS--Continued

	Page
Results	57
PCR Product Analysis	57
Hybridization Analysis	61
DNA Cloning and Sequencing	61
Discussion	69
4. SUMMARY	73
LITERATURE CITED	78

LIST OF TABLES

Table		Page
1.	Preliminary evaluation of two MT-RMV-V polyclonal antisera by indirect enzyme-linked immunosorbent assay (ELISA) using clarified leaf homogenates from oats infected with MT-RMV-V or NY-RMV or from healthy oats.....	38
2.	Preliminary evaluation of six monoclonal antibodies from two separate fusions against MT-RMV-V, NY-MAV, NY-RPV and NY-PAV infected and healthy plant sap by indirect enzyme-linked immunosorbent assay (ELISA).....	39
3.	Preliminary evaluation of antibodies from one mixed hybridoma cell line and six monoclonal cell lines against purified virions of Montana and New York RMV isolates of BYDV by indirect enzyme-linked immunosorbent assay (ELISA).....	39

LIST OF FIGURES

Figure		Page
1.	Purified virus preparations of the MT-RMV-V isolate of BYDV.....	25
2.	Ethidium bromide staining of a non-denatured nucleic acid gel.....	27
3.	Coomassie blue staining of a 14% SDS-PAGE protein gel.....	28
4.	Longitudinal section through phloem cells of oats, Avena byzantina (Koch) cv. Coast Black infected with BYDV MT-RMV-V.....	30
5.	Distinct clumps of MT-RMV-V virions in an infected oat cell.....	31
6.	Part of an inner cell wall callose deposit (C).....	32
7.	Numerous irregularly shaped vesicles (V) in a portion of an infected oat phloem cell.....	33
8.	Callose (C) occluded sieve plate pores (SP)....	34
9.	Rod like tubular membranes found in phloem cell cytoplasm.....	36
10.	Non-rigid tubules with varying diameters (arrows) found in phloem cell cytoplasm.....	37
11.	Primer base pairing locations on a BYDV Australian PAV isolate and BWYV.....	53
12.	PCR products using the Lu1-Lu4 oligonucleotide primers.....	58
13.	PCR products using the Pol1-Pol2 oligonucleotide primers.....	59
14.	PCR products using the Pol1-Lu4 oligonucleotide primers.....	59

LIST OF FIGURES--Continued

Figure		Page
15.	Restriction enzyme digests of Lu1-Lu4 PCR fragments	60
16.	Autoradiogram showing results of a Northern blot	62
17.	Autoradiogram showing the results of a Southern blot	62
18.	Nucleotide sequence of the Lu1-Lu4 PCR fragment of MT-RMV-V	63
19.	Enzyme restriction map of the Lu1-Lu4 PCR fragment of MT-RMV-V	63
20.	Nucleotide sequence of the MT-RMV-V Pol1-Pol2 PCR fragment	64
21.	Nucleotide sequence of the Lu1-Lu4 PCR fragment of NY-RMV	65
22.	Enzyme restriction map of the Lu1-Lu4 PCR fragment of NY-RMV	65
23.	Comparison of the deduced amino acid sequences of the MT-RMV Lu1-Lu4 genome fragment with the coat protein region of NY-RMV, NY-RPV, BWYV-FL1, PLRV-1 and Aus-PAV	67
24.	Comparison of the deduced amino acid sequences of the MT-RMV Pol1-Pol2 genome fragment with the RNA dependent RNA polymerse region of NY-RPV, BWYV-FL1 and PLRV-1	68

LIST OF ABBREVIATIONS

BLRV - Bean leafroll virus

BMV - Brome mosaic virus

BWYV - Beet western yellows virus

BYDV - Barley yellow dwarf virus

DEPC - diethyl pyrocarbonate

DNA - deoxyribonucleic acid

Lu1 - 5'-CCAGTGGTTRTGGTC-3' oligonucleotide DNA primer

Lu4 - 5'-GTCTACCTATTTGG-3' oligonucleotide DNA primer

MES - 2-[N-Morpholino]ethanesulfonic acid

MT-RMV-V - Montana BYDV-RMV isolate from Valier, Montana

NY-MAV - New York BYDV isolate vectored by Sitobian avenae (Fabr.)

NY-PAV - New York BYDV isolate vectored by Rhopalosiphum padi (L.) and S. avenae

NY-RMV - New York BYDV isolate vectored by Rhopalosiphum maidis (Fitch)

NY-RPV - New York BYDV isolate vectored by R. padi

NY-SGV - New York BYDV isolate vectored by Schizaphis graminum (Rondani)

PLRV - Potato leafroll virus

Pol1 - 5'-CGACTGCAGGGNTTYGAYTGG-3' oligonucleotide DNA primer

Pol2 - 5'-GCTGGATCCTGNSWRCARAAYTC-3' oligonucleotide DNA primer

RNA - Ribonucleic acid

TMV - Tobacco mosaic virus

ABSTRACT

A Montana barley yellow dwarf virus (BYDV) isolate, designated MT-RMV-V, was serologically indistinct from the NY-RMV type. It could be distinguished only by differential aphid vector transmission. Diameters of purified virions averaged 24.7 nm (S.D.=1.2 nm, n=559). Nucleic acid size was estimated by denaturing and non-denaturing gel electrophoresis with ethidium bromide staining. The relative molecular weight of the RNA was 1.7×10^6. The coat protein size was 21.7 Kd as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis with Coomassie blue staining. A cytological investigation of infected phloem cells by electron microscopy revealed that MT-RMV-V induced alterations were similar to those of a Manitoba RMV isolate and to those of an RPV isolate.

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CHAPTER 1

INTRODUCTION

Presently, barley yellow dwarf virus (BYDV) is the type member of the Luteovirus group. Members of this group are characterized by having isometric virions and positive sense single stranded RNA. Their capsid is composed of protein sub-units of a single molecular species. The viruses are phloem limited and are often found in low concentration in host plants (5,11,16,17,26,54,58,64,78,126,138,144,145). Members generally cause yellowing symptoms in the host, hence the name 'Luteo' for this group. All members are usually transmitted by aphids in a circulative, persistent, but non-propagative manner (17,39,42,78,144,148).

BYDV is found throughout the world in cereal growing regions, and is restricted to monocotyledonous plants. Other important Luteoviruses include beet western yellows virus (BWYV), a widespread virus infecting many dicotyledonous host species; potato leafroll virus (PLRV), found world wide but generally restricted to potatoes; and bean leafroll virus (BLRV) which normally infects legumes. A variety of strains have been associated with each of these members. In the past, viruses have been named by the host plant in which they were originally found. Because of this

naming system, synonymous names for a single virus are all too common. This has led to an ever increasing number of viruses in the luteovirus group. Casper (17) tried to narrow this list in 1988 by aligning like strains and viruses on the basis of serological relationships.

BYDV is the causal agent of a leaf discoloring and stunting disease of cereals that was first described in 1951 by Oswald and Houston in California (87). Five strains, or variants (49,50,101,112,117,119,120,121) or more recently isolates (113), of this virus have been reported. They were initially differentiated and named for the predominant aphid species which could vector them. The strains and their respective vector(s) are:

PAV - Rhopalosiphum padi (L.), Sitobion avenae (Fabr.)

MAV - S. avenae

SGV - Schizaphis graminum (Rondani)

RPV - R. padi

RMV - Rhopalosiphum maidis (Fitch)

The PAV strain is an aphid non-specific strain. Both aphid species, noted above, are capable of transmitting this strain.

Aphid vector specificity has been considered to be one of the most important methods used to differentiate and classify recently collected BYDV isolates. Gildow (39,40,41,43,44,45) has studied the complex aphid-BYDV relationship extensively using the electron microscope. In

thin sections of aphids, he discovered the importance of membrane barriers relative to the internal circulation of BYDV isolates in the aphid. The initial barrier is the lumen membrane of the hindgut. However this membrane is not highly selective and most BYDV isolates can pass through it in any given aphid species. The plasmalemma of the accessory salivary gland appears to be responsible for the observed aphid-virus vector specificity. It is this latter membrane that determines if a BYDV isolate will be transmitted by a particular aphid species. In one study, Gildow examined virus-membrane selectivity using S. avenae and the MAV, PAV, and RPV isolates. He found that all three isolates could pass through the hindgut lumen membrane, but only MAV and PAV could pass through the accessory salivary gland plasmalemma. No detailed studies of the exact receptor site(s) on the plasmalemma have been reported. Nor have aphid virus transport studies using BYDV isolates which are vectored by a variety of non-related aphid species been reported.

Classification of the BYDV isolates was made in order to explain the observed similarities and differences within the group. The PAV, MAV, and SGV isolates, are considered to be in sub-group I and the RPV and RMV are considered to be in sub-group II. These initial groupings were made based on serology (50,107,110) and pathological ultrastructural changes induced in the host cells (45,47). Analyses of ds

RNA profiles (41,136,137) and nucleic acid sequences (141,142) have lent support to these divisions. The genomic organization of the six open reading frames appears to differ in the two sub-groups (68,78). The two overlapping non-structural genes are located within the 5' half of the genome for both sub-groups. But they encode 39 Kd and 60 Kd products for PAV isolates and 70 Kd and 69-72 Kd products for BWYV, PLRV and NY-RPV (78,141). In the PAV and MAV isolates the coat protein gene, with an internal virus-linked protein gene, is located more centrally in the genome. In RPV these genes are located more toward the 3' end of the genome. In addition, the putative protease gene is located at opposite ends of the genome for each sub-group. With PAV and MAV the protease gene is located at the 3' end, but in RPV it is at the 5' end.

BYDV can cause periodic epidemics resulting in significant crop loss (3,21,46,48,150). Often plants infected with a single BYDV isolate display physiological stress (2,29,30,31,33,36,63,65,66,67,73,129) which results in not only decreased yields, but increased plant susceptibility to other pathogens (130). Multiple infections of plants by two or more isolates usually reduce yields more than do single infections. Synergistic effects between the MAV and RPV isolates, as well as between the RMV and PAV isolates have been reported (46). In addition, one BYDV isolate may act as a helper virus to allow the

subsequent uptake of a non-transmitted isolate by a particular aphid species (103). The phenomena of helper viruses and heterologous encapsidation, ie. the coat protein of one isolate encapsidating the nucleic acid of another isolate, greatly enhances the possibility of increased spread of a more virulent, but perhaps less represented strain within a crop. W.F. Rochow observed that in NY grain crops, the prevalence of vector-specific isolates decreased while the prevalence of a vector non-specific isolate increased during a 10 year period (102,105,106,109,114,115). Although no other U.S. geographic region has been studied as extensively, the NY study suggests that BYDV isolate prevalence could change over time. For example, it is possible that a less virulent isolate could be displaced by a more virulent one in a region.

The most common control measures are: the use of selected planting dates, the use of tolerant cultivars and the application of pyrethroids and organophoshates to control aphids. Altering crop planting dates avoids having young, susceptible plants available to migrating, infective aphids (21,48,62,71,97,99,132,150). Breeding for resistance to BYDV has had limited success (56,93). Cereal cultivars which contain genes which condition for tolerance to BYDV are found in barley and oats, but these cultivars are often restricted in their growing regions. Some cereals are

naturally resistant to aphid feeding (84), but breeding experiments with these plant cultivars have been limited.

Because cereal crops are annual, the virus must be introduced to the plants each year by the aphids. It has been suggested that one host species may act as a reservoir of the virus for secondary spread (13). Corn was suggested as one such possible reservoir. Brown et al. (13) suggested that a Washington PAV isolate may move from wheat to corn via S. avenae and then from corn to wheat via R. padi. However, studies by Blackman et al. (9,10) and Brown and Blackman (14) suggest that movement from one crop to another, such as from corn to barley, by corn leaf aphids (R. maidis) is not probable. V.F. Eastop and R.L. Blackman (British Museum of Natural History, London England) analyzed the karyotype of corn leaf aphids collected from corn, spring barley and wheat. They determined that aphids collected from spring barley had a 2n=10 karyotype whereas aphids obtained from corn had a 2n=8 karyotype. Both of these aphid types were capable of transmitting BYDV RMV-like isolates efficiently (15). A clone of W.F. Rochow's NY biotype of R. maidis had a 2n=8 karyotype, indicating that it was probably originally collected from corn. A 2n=8+1 karyotype was reported for aphids collected from wheat plants in some parts of the world (10). It has not been reported that the three distinct aphids can switch their feeding from corn to spring barley or to wheat in nature

(9,15). This finding would eliminate one possible mechanism by which BYDV can overwinter and spread between two different crops.

The determination of multiple karyotypes for individual aphid species has been reported previously by Blackman (10). Often the chromosome change does not result in a gross morphological change in the aphid, and thus the splitting of one species into two or more species has not been recommended. However, it is unknown what genomic information is carried on the extra chromosome and if it has any influence on an aphid's ability to vector viruses. It is also unknown what, if any, genetic information is lost for aphids which have reduced chromosome numbers.

Yount and Carroll (149) reported the presence of serologically unique RMV-like isolates collected in Montana from 1978-1981. These original isolates failed to react to W.F. Rochow's bank of NY-BYDV antisera in double antibody sandwich enzyme-linked immunosorbent assay. This was a significant departure from results normally obtained for BYDV isolates. Generally field isolates react with an antiserum to at least one of the NY type isolates (20,22,23,25,27,32,59,89,107,108,134). Because of this serological difference, a more thorough investigation of MT RMV-like isolates was undertaken. During 1985 and 1986, a second set of BYDV field isolates was collected. Five RMV-like isolates, based on aphid transmission data, were

retained for further characterization. Three of the MT RMV-like isolates were efficiently transmitted by corn leaf aphids and by greenbugs (15). When these isolates were tested against NY polyclonal antisera, they reacted positively with NY-RMV immunoglobulin but not with NY-SGV, NY-RPV, NY-PAV or NY-MAV immunoglobulins in enzyme-linked immunosorbent assays (15). Whether the original RMV-like isolates present in Montana changed over time, or if new isolates were moved into the state by migrating aphids, or whether the antiserum produced recently is more effective at detecting RMV-like isolates is uncertain.

Because of the anomaly in aphid vectors for the MT RMV-like isolate, we hypothesized that other properties of the MT isolate may differ from those of the NY-RMV isolate. The purpose of this research was to purify one of the MT RMV-like isolates and characterize it chemically and physically. A second objective was to examine infected phloem cells for cytopathological alterations. A third objective was to produce antibodies specific for the MT RMV isolate. Our final objective was to partially sequence the coat protein gene, since this gene may be involved in aphid vector specificity. If the MT and NY isolates were to be dissimilar genomically, we hypothesized that it would be in the coat protein region. By characterizing an RMV-like isolate, we hoped to gain basic knowledge that could help

elucidate the taxonomic relationships and classification within the BYDV group.

CHAPTER 2

BIOLOGICAL INVESTIGATION

Introduction

Barley yellow dwarf virus (BYDV) is the causal agent of a dwarfing and leaf discoloring disease of gramineous hosts (87,120). Four aphid specific virus isolates and one aphid non-specific isolate of BYDV have been described by Rochow (113,118), Gill (49,50), and Rochow and Muller (111). Since that time, most workers worldwide have compared their local field isolates of BYDV to the five New York (NY) isolates of Rochow (1,13,27,77,94,112,131). Although some of those local isolates were characterized and eventually designated according to the location in which they were collected; i.e. IL-PAV (24), P-PAV (55), and CA-RPV and CA-PAV (22), each was similar to at least one of the NY isolates. These similarities included, but were not limited to, ds RNA profiles, aphid vector specificity, serology and cross protection.

Five Montana (MT) BYDV isolates collected in 1986 were found to be RMV-like on the basis of vector specificity. Upon further investigation, it was determined that the MT RMV-like isolates were similar to the NY-RMV isolate

serologically, but were more virulent in Avena byzantina (Koch) cv. Coast Black oats than either NY-RMV (15). Additionally, the MT isolates were transmitted by both Rhopalosiphum maidis (Fitch)(corn leaf aphid) and Schizaphis graminum (Rondani)(greenbug). Two MT biotypes of R. maidis, one having a karyotype of 2n=10 and the other having a karyotype of 2n=8, vectored three MT RMV-like isolates with average transmission efficiencies of 41% and 59% respectively (9,15). By comparison, the NY biotype of R. maidis, having karyotype 2n=8, had only an average transmission efficiency of 29% for the three MT RMV-like isolates. More importantly, the NY biotype of S. graminum had a relatively high average transmission efficiency of 19% with the same three MT RMV-like isolates (15). S. graminum rarely transmits NY-RMV (16).

Although it is unusual for two serologically indistinct BYDV isolates to have different aphid transmission properties, it is not without precedent. Creamer and Falk (22) reported a California (CA) RPV isolate which was serologically indistinct from the NY-RPV type, and yet, was non-specifically transmitted by three aphid species. NY biotypes of Rhopalosiphum padi (L.)(oat-birdcherry aphid) and Sitobion avenae (Fabr.) (English grain aphid) as well as CA biotypes of S. avenae and S. graminum were able to vector the CA-RPV isolate.

Research devoted to RMV isolates has been limited mostly to aphid transmission, cross protection and various serological investigations. Research on the purification and characterization of RMV virions was needed in order to advance the basic understanding of these isolates. The purpose of this study was to purify and characterize one of the MT RMV-like isolates. A cytological investigation of infected oat leaves was undertaken to characterize one of the many biological properties of this isolate. A final objective was to develop MT-RMV specific polyclonal and monoclonal antibodies for use in diagnostic testing.

Materials and Methods

Host Plant

Three seeds per 10 cm plastic pot of *Avena byzantina* (Koch) cv. Coast Black oats were sown in soilless Sunshine mix and maintained for two weeks prior to inoculation in a Percival growth chamber, programmed for a 12 hr photoperiod (10,000 lux) and 24 C temperature. Plants were watered every two days and were fertilized with an all-purpose commercial fertilizer every two weeks.

Aphids

Rhopalosiphum maidis (corn leaf aphid) colonies were supplied by W.F. Rochow in 1986, or collected from spring

barley and corn fields in Montana during 1985 and 1986. Colonies were designated as follows:

NY-RM (Derived from the NY biotype of R. maidis)

MT-RM SB (MT R. maidis from spring barley)

MT-RM C (MT R. maidis from corn)

Non-viruliferous colonies were increased on Hordeum vulgare (L.) cv. Klages within nylon mesh cages. Cages were 13 cm in diameter and 50 cm high. Aphid colonies were kept in a Conviron growth chamber programmed for 20 C and 15 hr photoperiod (10,000 lux). New colonies were started every month with first instar nymphs from an old colony or from three to five isolated, apterous adults. Six to twelve aphids were used for each pot containing 50 young Klages barley plants.

Virus

An RMV-like isolate of BYDV was obtained in 1986 from infective aphids feeding on spring barley located near Valier, MT. This isolate was subsequently characterized on the basis of its aphid transmission and serological characteristics (15). It has been maintained by serial aphid transfer in California Red oats (A. sativa) and Coast Black oats. This BYDV isolate has been designated as MT-RMV-V. The RMV isolate from W.F. Rochow's New York collection, designated NY-RMV, was obtained in 1986 and was similarly maintained.

The MT-RMV-V isolate was propagated by allowing healthy, nonviruliferous aphids to feed on detached, MT-RMV-V infected oat leaves for one to two days followed by a five to six day inoculation access feeding on two to three week old healthy oat plants (116). In excess of 20 presumed infective aphids, per plant, were given the opportunity to feed. Plants were enclosed by 8 cm x 30 cm acrylic and nylon mesh cages to prevent escapes and cross contamination. Plants were placed in the green house programmed for 24 C and a 15 hr photoperiod (10,000 lux) or in a Conviron growth chamber programmed for 20 C and a 15 hr photoperiod. Plants were fumigated for 30 minutes with Vapona (Diclorvos, Diamond Shamrock Corp.) to kill the aphids. Fumigated plants were then returned to the green house or the growth chamber, where they developed symptoms three to five weeks after inoculation. These plants were either maintained as stock plants or were harvested and stored at -20 C.

Virus Purification and Assay

The Valier RMV isolate of BYDV was purified using a method modified from those described by Hammond et al. (55), D'Arcy et al. (24), Christie et al. (18), Lee and Davis (74) and S.Gray (personal communication). The buffer system used and a reduction in the number of low speed centrifugations were the major modifications in the purification method reported here. Generally, 50 g of starting material was

used, but as little as 35 g or as much as 100 g was used without having to alter the procedure or time involved for processing. All but completely discolored and dried leaves were harvested from diseased plants for virus purification. Leaves, and occasionally roots and stems, were ground in excess liquid nitrogen with a mortar and pestle, then ground to a finer powder in a Sorvall Omni-Mixer. Cold buffer (0.1 M 4-morpholineethanesulfonic acid (MES), 0.02 M ethylenediaminetetraacetic acid (EDTA), 0.5% sodium sulfite, pH 6.2) was added 1:2 (w/v) and the mixture was homogenized for three to five minutes on ice. Extractase P20X enzyme (Finnsugar Biochemicals, Rochester, NY) was added at the rate of 0.02 g/ml mixture. After blending, the mixture was allowed to stand on ice 30 minutes and then 2.5 hr at room temperature. The mixture was given periodic homogenization for 15 seconds during this time. Triton X-100 was added at the rate of 5 ul per ml of total volume of homogenized mixture. The mixture was slowly stirred at room temperature for 30 minutes. A 1:1 mixture of cold chloroform and n-amyl alcohol was added to the homogenate at the rate of 3.3 ml per 5 ml of homogenate, blended rapidly for 30 seconds, and then stirred slowly at room temperature for 30 minutes.

This mixture was centrifuged in a Sorvall SS34 rotor at 12,100 x g for ten minutes, at 4 C. The supernatant was filtered through Kimwipe paper tissues. One-third volume of a 30% PEG (ave. mol. wt. 7000-9000), 0.6 M NaCl stock

solution was added to the supernatant. After shaking vigorously, the supernatant was allowed to stand overnight, at 4 C. The supernatant was centrifuged in a Sorvall SS34 rotor at 27,000 x g for ten minutes, at 4 C. Pellets were resuspended in 4 to 5 ml MES buffer, layered onto a 30% sucrose pad and centrifuged in a Beckman Ti80 rotor at 100,000 x g for 3 hr, at 4 C. Pellets were resuspended in 1 ml MES buffer for 1 hr and then layered on a sucrose gradient (4,12). The gradient was made by layering 8.0 ml each of 10, 20, 30, and 40% sucrose solutions in MES buffer and allowing the sucrose to diffuse at room temperature for 4 hr so the gradient would become continuous. The gradient was centrifuged in a Beckman SW28 rotor at 85,000 x g for 3 hr, at 4 C. After discarding the first 2 to 3 ml collected, four 8.0 ml fractions were collected, in sequence, from the top of the centrifuge tube with an Isco Model 640 fraction collector connected to an ISCO Model UA-5 absorbance monitor. Fraction F-1 was the first 8.0 ml collected, fraction F-2 was the second 8.0 ml collected, and so forth. Fractions were centrifuged in a Beckman Ti80 rotor at 150,000 x g for 3 hr, at 4 C. Each pellet was resuspended in 200 to 300 ul of buffer and assayed by ultraviolet absorbance spectroscopy to estimate virus concentration and purity and by transmission electron microscopy. Fractions were then stored at -20 C until further use.

Spectrophotometric readings of purified virus preparations were determined in a Beckman Model DU 50 spectrophotometer. Yields were estimated with the following formula and extinction coefficient of 8.0 supplied by S.M. Gray (personal communication).

$$mg/ml = [(A_{260} - A_{320}) \times \text{dilution factor}] / 8.0$$

Yields were then converted to mg/Kg of plant starting material. The average $A_{260/280}$ value was calculated, after correcting for light scattering (A_{320}) (4,28).

Formvar and carbon coated 300 mesh grids were prepared according to Christie et al. (18). Ten microliters of each centrifuge fraction (F-1,F-2,F-3 and F-4) from virus infected or healthy plant preparations were placed on individual grids for one to two minutes. Grids were washed with 1 ml filtered, distilled water containing 250 to 300 ug/ml bacitracin (Sigma), as a wetting agent, and then stained for 5 seconds with a 2.0% uranyl acetate solution in water which also contained 250 to 300 ug/ml bacitracin. After staining, grids were dabbed dry without further washing. Samples were observed at 60 kV with a Zeiss EM 10CA electron microscope. Virion measurements were taken from electron micrographs using a Zeiss Interactive Digital Analysis System (ZIDAS).

Estimation of Virion Chemical Components

All Luteoviruses have been shown to be single stranded RNA viruses (17,78). Based on aphid specificity, disease symptoms, and virion morphological similarities between the MT-RMV-V isolate and the other BYDV isolates, the MT-RMV-V isolate was assumed to contain RNA. The size of the virion RNA was estimated by three different methods. In the first method, RNA was extracted from those fractions with the highest numbers of virions, as revealed by electron microscopic assay and A_{260} estimation of virion concentration. One-half volume of buffer (0.2 M glycine, pH 9.5, 0.2 M NaCl, 20 mM EDTA), 80 ul of 20% sodium dodecyl sulfate (SDS) (Gallard-Schlesinger, Carle Place, NY), and 16 ul of 20 mg/ml Proteinase K (Promega, Madison, WI) were added per milliliter of virion starting volume. The mixture was vortexed briefly and then incubated at 37 C for 30 min. Phenol was added at the rate of 2 ml/ml starting volume, and the mixture vortexed 1 minute before it was centrifuged at 12,000 x g for 30 min. at 4 C. RNA was precipitated from the aqueous phase with 0.1 volume of 3 M sodium acetate (NaOAc) and 3 volumes of cold, 100% ethanol (EtOH). The mixture was held at -70 C for 15 min. and then centrifuged at 12,100 x g for 30 min. at 4 C. The pellet was resuspended in 300 ul of 0.3 M NaOAc and RNA reprecipitated with 2.5 volumes of cold, 100% EtOH and stored overnight at -70 C. Viral RNA was

microfuged, washed, dried and resuspended in 20 ul of diethyl pyrocarbonate (DEPC) treated water as per accepted protocols (86,123,143). Virion RNA size was determined by 1% LE agarose (SeaPlaque, FMC BioProducts, Rockland, ME) electrophoresis after denaturation with formamide and formaldehyde (123). The entire 20 ul sample, approximately 21 ug, of resuspended RNA was loaded into each well. The gel was stained in 0.5 ug/ml DEPC treated water of ethidium bromide. A 0.24-9.5 Kb RNA ladder (BRL, Gaithersburg, MD), brome mosaic virus (BMV) RNA (Promega) and tobacco mosaic virus (TMV) RNA (52) were used as standards. BMV contains four nucleic acids in its virion. The relative molecular weights (Mr) of its RNA are Mr 1.0×10^6, Mr 1.0×10^6, Mr 0.7×10^6 and Mr 0.35×10^6. TMV has a nucleic acid Mr of 2.0×10^6. The commercial BMV marker was prepared according to Sambrook et al. (123) for all RNA gels.

In the second method, the following were combined: 25 ul of purified virus , 2 ul of 2-mercaptoethanol, 10 ul of formaldehyde, 10 ul of formamide and 1 mg of SDS. This mixture was incubated for 45 minutes at 65 C. Thirty-five microliters of each sample, approximately 26 ug of RNA, were loaded and then run at 40 V on a 1% agarose, formaldehyde denaturing gel according to Sambrook et al.(123). The gel was stained in either 1.5 ug/ml ethidium bromide in DEPC treated water or 0.5 ug/ml acrydine orange in DEPC treated water.

For the third method, approximately 21 ug of virion RNA was prepared by incubating purified virions for 1 hr at 37 C in dissociation buffer (0.1 M Tris, pH 8.0, 5% 2-B mercaptoethanol, and 4% sodium dodecyl sulfate) (57,58). Samples were prepared for electrophoresis in 1% LE agarose, 1/2x TBE (0.089 M Tris-borate, 0.89 M boric acid, 0.02 M EDTA), 150V for 2 hr and stained afterward in 0.5 ug/ml of ethidium bromide in DEPC treated water. Standard markers were BMV and TMV RNA.

Viral coat protein molecular weight of purified virions was determined by 14% discontinuous SDS-polyacrylamide gel electrophoresis (58,85), 30 mA for 3 hr, and visualized by both Coomassie blue and silver staining (BioRad Labs, Richmond, CA). One hundred microliters of concentrated MT-RMV-V virions, approximately 53 ug, were solubilized in 4% SDS, 5% 2 B-mercaptoethanol, 20% glycerol, 0.1 M Tris, pH 8.0 and boiled 5 min. prior to loading. An 80 ul sample was loaded into each lane. Low molecular weight standards (BioRad) and TMV coat protein were used as markers. Purified NY-RMV was included as an additional marker and for comparison purposes.

Thin Section Electron Microscopy of Infected Plant Tissues

Coast Black oats were inoculated with MT-RMV-V by infective R. maidis at 14 to 16 days post sowing and allowed to feed as described earlier. At 21 to 28 days post

inoculation the outer leaf margins, of leaf sections showing slight chlorotic mottling, were harvested and prepared for examination with the Zeiss EM 10CA electron microscope.

Leaves were cut into approximately 1 mm^2 pieces and fixed overnight at 4 C in 3% glutaraldehyde in 0.2 M sodium potassium phosphate (NaK_2PO_4) pH 7.2. Post fixation was in 2% osmium tetroxide (OsO_4), followed by dehydration in a 70-100% ethanol series. Samples were treated with propylene oxide and then infiltrated with Spurr's epoxy resin. Ultrathin sections were cut with a diamond knife (DuPont, Wilmington, DE) on a Reichart OM-U2 ultramicrotome and doubled stained with uranyl acetate followed by Reynold's lead citrate. Samples were observed at 60 kV.

Polyclonal Antisera Production

Polyclonal antisera to the MT-RMV-V isolate of BYDV were raised using two, young adult New Zealand white rabbits. Fractions containing purified, intact virions, as per electron microscopic assay, were used as the antigen source. Initially, 1.0 cc of virus containing fractions, approximately 210 ug of virus per ml, were mixed with an equal amount of Freund's Complete adjuvant (Sigma). Subsequent booster injections using a 1:3 ratio of freshly prepared virus containing fractions and Freund's Incomplete adjuvant (Sigma) were made at 2 week intervals for 6 weeks. Blood was drawn during the intervening weeks.

Antisera were tested for specificity to the antigen by indirect ELISA (19,76) using clarified homogenates from virus infected leaves as test samples. The homogenates were produced by following the virus purification procedure through the resuspension of the PEG/NaCl precipitation step. One hundred microliters of homogenate sample were placed in each well in Immulon II ELISA microtiter plates (Dynatech, Rockville, MD). The homogenate was incubated for 3 hr at room temperature. After washing in PBS + 0.5% Tween-20, the wells were blocked with 0.2% egg albumin before the addition of 100 ul of each polyclonal antiserum individually to each well. Incubation of this step was for 4 hr at room temperature. Goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase were used to detect positive samples.

Monoclonal Antibody Production

MT-RMV-V antigen was purified following the method of D'Arcy et al. (24). A 1:1 ratio of antigen, approximately 100 ug, and Complete Freund's adjuvant (Difco, Detroit), total volume equalling 0.5 cc , were injected intra-peritoneally into each of 4 six week old, male BALB/c mice. Booster injections were given intravenously, in the tail, 6 weeks after mice were immunized.

A three week old, female BALB/c mouse was anesthetized and its thymus surgically removed. Thymocytes were washed and teased apart in a glucose sodium-phosphate buffer,

centrifuged at low speed and resuspended in HAT medium [Dulbecco's Modified Eagle Medium (GIBCO), 100 uM hypoxanthine, 0.4 uM aminopterin, 16 uM thymidine (Sigma) and 10% heat inactivated horse serum (Hyclone, Logan, UT)]. Feeder cells were plated at 2 x 10^5 cells/well and incubated in 24-well, flat bottomed culture plates (Corning, Corning, NY) at 37 C.

Three days after boosting immunized mice, spleens were aseptically removed from 2 mice and were used in separate fusions with P3-X63-Ag8.653 BALB/c plasmacytoma myeloma cells (American Type Culture Collection, Rockville, MD) (38,51,69,72,81,135). One milliliter of hybridoma culture was placed into each of 144 tissue culture wells containing 1 day old thymocyte feeder cells per fusion. Hybridomas were incubated at 37 C for three days without feeding. Every third day, 1/2 of the media was aspirated and fresh HAT media added. After 12 days HT media, HAT media without the aminopterin, replaced HAT media.

Hybridomas were initially screened by indirect ELISA for antibody specificity (see above for procedure). Goat anti-mouse antibodies conjugated to alkaline phosphatase, at a 1/500 dilution, were used to detect positive samples. Positive hybridoma wells were cloned by limiting dilution cloning and again screened by indirect ELISA for monoclonal antibody production. When screened against purified virus

preparations, 50 ul of each virus preparation was added per well, and 100 ul of each monoclonal supernatant was used.

Results

Virus Purification and Assay

Determination of average virus yield was based on data from 11 purifications. Spectrophotometric readings of purified virus preparations taken at A_{260} , A_{280} and A_{320} indicated that the virus yield ranged from 1.47 mg/kg to 7.21 mg/kg with a mean yield of 4.2 mg/kg of infected, plant starting material (S.D.= 2.04 mg/kg, n=11). The $A_{260/280}$ absorbance ratio was 1.84 (S.D. = 0.23, n=10) for the MT-RMV-V isolate based on spectrophotometric readings from 10 virus purifications.

MT-RMV-V virions were purified from all gradient fractions except F-1, although virions were observed in that fraction. In general, F-2 or F-3 fractions had the highest concentration of virus particles and were the cleanest preparations, as per electron microscopic assay. Most virions appeared isometric in shape, although some looked swollen or ruptured (Fig. 1.). An arrow in Fig. 1c points to a ruptured virion. Swollen virions are marked with an "*" in Fig. 1. A total of 559 virions were measured from 11 virus purifications. Virion diameters ranged from 23.0 nm to 27.0 nm with an average of 24.7 nm (S.D. = 1.2 nm).

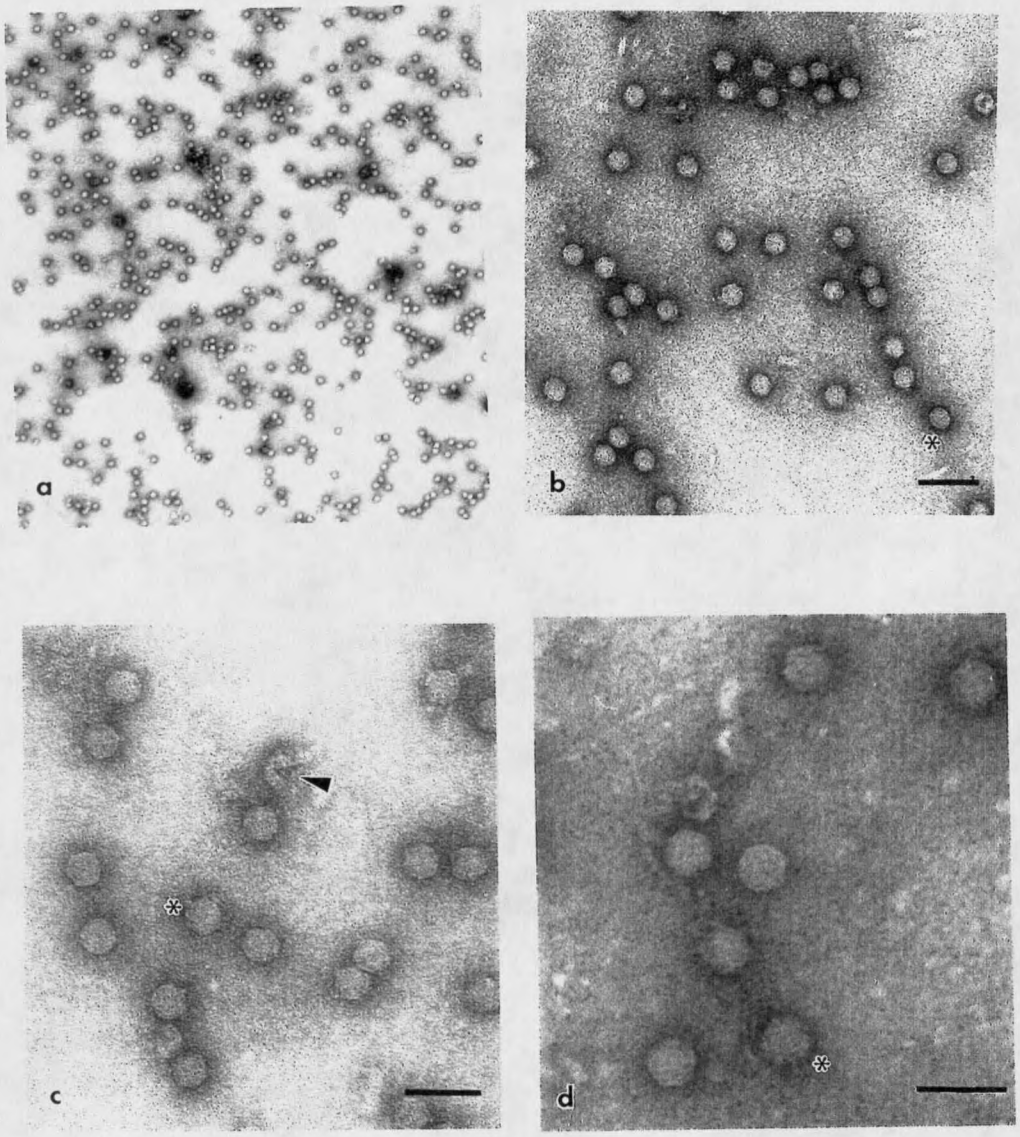

Fig. 1. Purified virus preparations of the MT-RMV-V isolate of BYDV. a) x 27,500 b) x 109,400 c) x 175,000 d) x 218,700. Bar represents 50 nm.

Estimation of Nucleic Acid Size

Electrophoresis of extracted viral RNA in formaldehyde denaturing gels was unsuccessful. No viral RNA bands could be detected. Using intact virions, dissociating the nucleic acid from the coat protein and then denaturing the viral RNA was slightly more successful. An extremely faint band was detected with an apparent Mr of 1.70×10^6. This size correlates to approximately 5.1 Kb in length. A single RNA band was observed with an apparent Mr of 1.65×10^6 in the non-denaturing gel (Fig. 2.). This size would correlate to having approximately 4955 nucleotide bases in the virus genome.

Estimation of Coat Protein Size

A major polypeptide band was observed having an apparent Mr of 21.0 to 21.7 Kd (Fig. 3.). Occasionally, 1 or 2 smaller minor bands with apparent Mr's of 20.6 Kd and 18.7 Kd were seen when SDS-PAGE gels were stained with silver. A polypeptide doublet with apparent molecular weights of 59.0 Kd and 60.0 Kd were also seen.

Cytopathology of Infected Tissues

Sieve elements, phloem parenchyma and companion cells contained virions and/or displayed various cytopathological alterations induced by virus infection in the plant. Virions were detected in only a few types of cells. Frequently they were located in electron dense areas